

Session 3

Cloning Overview & Polymerase Chain Reaction

Learning Objective:

In this lab exercise, you will become familiar with the steps of a polymerase chain reaction, the required reagents for a successful reaction and the concept of making a “master mix.”

Introduction

Polymerase chain reaction (PCR) is perhaps the most important development in the field of molecular biology in the past 50 years. PCR allows the scientist or engineer to ***amplify*** DNA ***in vitro***. Usually only a small region of the ***template*** DNA, called the ***target sequence***, is amplified. Using PCR, many copies of the desired target sequence can be made leaving only small amounts of the undesired portion of the template. PCR has many uses, some of which include: cloning, sequencing, mutagenesis, and quantifying DNA or RNA.

Background

Cloning a Gene

Cloning a gene is the process by a region of DNA encoding a gene of interest is put into another setting. Typically when a gene is cloned, the DNA is amplified from a target source and put into a ***vector***. The vector is then put into the cell of interest. Using the techniques described last week, the cell with the cloned gene is selected and isolated. At that point, the researcher can study whatever topics are of interest with the use of the cloned gene.

In the remainder of this laboratory class, we will go through all of the steps in cloning a gene and characterizing the expression of that gene in *Escherichia coli*. The gene we will be cloning encodes for a LacZ α -GFP fusion protein. The LacZ α portion of the gene encodes for part of an enzyme called betagalactosidase. At the end of the project, we will determine how much betagalactosidase is in strains with different expression systems.

Today we will be taking the first step in our cloning process. This involves the PCR amplification of the LacZ α -GFP gene from our template DNA. The PCR product will also include the ribosome binding site (RBS) required for translation of the gene. At the end of this step, we will have plenty of the DNA to complete the cloning process. PCR is explained below in more detail.

After the PCR is completed, we will purify vector DNA and ***restriction digest*** the vector and PCR DNA (Session 4). This process cuts the DNA and makes it possible to join the PCR DNA with the vector DNA. After the DNA has been cut, we will purify the DNA

of the proper length using **gel electrophoresis** (Session 5). The segments of DNA will be joined using **ligation** and **transformed** into *E. coli* cells (Session 6). Colonies containing cloned cells will be stored and **sequenced** (Session 7). We will then compare the expression and function of the cloned gene in different expression systems and conditions (Sessions 8 & 9).

Reagents of the Polymerase Chain Reaction

PCR requires several various reagents. First, you need the template (pattern) DNA which contains the target sequence to amplify. The template can come from different sources of DNA including: previous clones, isolation from an organism, isolation from the environment, or chemical synthesis. The origin of the template usually depends on the intended application and availability.

In order to make new DNA, you need to include the building blocks in the reaction mixture. DNA (deoxyribonucleic acid) is composed of **deoxynucleoside triphosphates (dNTPs)**. These building blocks contain the nitrogenous base (responsible for encoding the genetic information), a deoxyribose sugar (the main component of the polymer backbone), and a triphosphate chain (which stores the energy required for the polymerization reaction and provides the link between the sugars). Since there are four bases used in the construction of DNA, adenine (A), guanine (G), thymine (T), and cytosine (C), there are four dNTPs in the mixture (dATP, dGTP, dTTP, and dCTP).

The nucleotides must be assembled by a **DNA polymerase** enzyme. The DNA polymerase enzyme uses the template DNA to add the appropriate base (the complementary pairing base to the template, A → T, T → A, C → G, G → C) to the growing DNA chain. The polymerase enzyme requires a certain set of conditions to be operational. To achieve the appropriate concentrations of salt, metal ions, and pH, a buffer solution is added to the reaction mixture. The polymerase must also be suited for operation at the appropriate temperature.

In order to start the PCR reaction, you must include short **oligonucleotides** (about 20-base (-nucleotide) long pieces of DNA) called **primers**. The primers are crucial to the success of the reaction. The primers provide a suitable location for the polymerase enzyme to begin working and indicate the ends of the of the target sequence. Because the primers dictate the target sequence, they must be designed for each PCR with the template in mind. Two primers are required for a conventional PCR amplification reaction. These primers work on opposite strands of the DNA and from opposing ends of the target sequence. Remember that DNA has two strands and that each strand is directional, with a beginning (termed 5') and end (termed 3'). The two strands are anti-parallel so that the 5' of one strand is by the 3' of the other strand. Thus, each primer corresponds to the 5' region of one of the strands of the target sequence.

Steps in the Polymerase Chain Reaction

PCR is done in *cycles*. A reaction typically consists of 25-30 cycles. The perfect completion of a single cycle results in doubling the number of DNA copies from the beginning of the cycle. So, if you start with 1 piece of double-stranded DNA, after one cycle you will have 2 pieces, after two cycles you will have 4, after three cycles you will have 8, and so on.

There are three steps to a PCR cycle. The first step is called *denaturing*. In the denaturing step, the temperature is raised to about 95 centigrade. This allows the double stranded template DNA to separate into two individual strands. The second step is called *annealing*. In the annealing step, the temperature is quickly cooled to about 55 centigrade. This allows the primers to attach to the individual strands of template DNA. The third step is called *elongating* or *extending*. The extending step is performed at about 72 centigrade. In the extending step, the DNA polymerase adds dNTPs to the primer DNA fragment according to the template strand pattern, making the new DNA strand longer. This happens until the polymerase reaches the end of the template strand and no longer has a template for making DNA. At the conclusion of the third step, the original double-stranded DNA has been turned into two double-stranded copies of DNA, each with a single strand of the original DNA. The process can now be repeated over and over again to make many more copies of the DNA.

Session 3: Pre-Laboratory Exercises

Name: _____

Date: _____

- 1) Read about subcloning (a good place to start is here:
<http://en.wikipedia.org/wiki/Subcloning>).
- 2) What is the purpose of a vector in cloning?
- 3) What is ligation? Transformation?
- 4) Go through the online tutorial about DNA structure and synthesis
(<http://depts.washington.edu/mlab/biologyTutorial/>)
- 5) What is a dNTP?
- 6) Temperatures are very important in the PCR process
 - a) Why do we heat the DNA in the first step?
 - b) What happens when the DNA is cooled back down in the second step?
 - c) What is the main factor for the temperature in the third step?
- 7) What happens to the number of DNA molecules after 1 round of PCR?
- 8) How many types of primers are needed for a PCR? Why?

Laboratory Protocol

The exercise: You will be amplifying a Beta-D-galactosidase/GFP *fusion protein* from a previously cloned template. You will be amplifying the gene with primers containing BioBricks restriction sites and a *ribosome binding site* on the forward primer. The product will be later combined with a promoter and the pSB4A5 vector in a BioBricks assembly process.

Materials: PCR tubes and caps
 1.5 mL microcentrifuge tube
 Sterile Deionized Water
 Template DNA
 Forward Primer
 Reverse Primer
 dNTPs
 Reaction Buffer
 Taq DNA Polymerase

Equipment: Vortexer
 Thermocycler

Protocol

1. To one of the PCR tubes, add 1 uL of the template DNA. To the other PCR tube at 1 uL of sterile water. Make sure to label the tubes indicating either LacZ or water and your group initials.
2. In a clean, sterile 1.5 mL microcentrifuge tube mix the following PCR reaction reagents. Listed below are the amounts for one 50 uL reaction, prepare a mix for three reactions. Mix well by vortexing.

5 uL 10X Buffer
1 uL dNTPs
2.5 uL Forward Primer
2.5 uL Reverse Primer
37.5 uL Water
0.5 uL Taq DNA Polymerase*

*Make sure to add polymerase last. The instructors will bring you polymerase when you are ready to add it.

3. Add 49 uL of the master mix to each of the PCR tubes. Put the caps on the tubes and mix. Collect the liquid in the bottom of the tube by gently tapping the tube against the benchtop.

4. Put the PCR tubes into the thermocycler and record which position they are in.
5. Set the thermocycler to the following settings:
 - a. Initial Denaturing at 95° C for 2 minute
 - b. Denaturing at 93° C for 40 seconds
 - c. Annealing at 55° C 30 seconds
 - d. Elongation at 72° C for 1 minute
 - e. Final Elongation at 72° C for 10 minutes
 - f. Hold at 4° C

Cycle steps b-d for 30 cycles

Session 3: Post-Laboratory Exercises

Name: _____

Date: _____

- 1) What does the gene you amplified today do? (Hint: look back to Session 2 and do some searching on the internet)

2) PCR Calculations

Assuming you started with 1 double stranded piece of DNA...

- a. How many molecules are there after:
 - i. 5 cycles
 - ii. 10 cycles
 - iii. 30 cycles
 - iv. n cycles
- b. If the PCR process is only 50% efficient (i.e. only half of the strands are successfully copied), if you start with 100 copies of DNA, how many are present at the end of
 - i. 1 cycles
 - ii. 2 cycles
 - iii. n cycles
- c. If your target DNA sequence is 1,000 basepairs long what is the maximum number of **double-stranded** DNA molecules that could be made starting with 50 microliters of a 200 micromolar solution of dNTPs? (Hint: You will need to use Avogadro's number. Start by figuring out how many dNTPs you have in the solution)

3) Where does “Taq” polymerase get its name? What is special about the organism it comes from that allows it to be used for PCR?

4) Based on the sequence below, design 10-base primers (please write in standard 5' to 3' notation) to amplify:

5' – ATGTGATGCC TAGCTTAGCT ACCTGTAAGA GGCATTACGT – 3'
3' – TACACTACGG ATCGAATCGA TGGACATTCT CCGTAATGCA – 5'

a. The entire sequence

b. The left half of the sequence

c. The right half of the sequence

References & Additional Reading

Wikipedia:

Polymerase Chain Reaction http://en.wikipedia.org/wiki/Polymerase_chain_reaction

Deoxyribonucleoside Triphosphate <http://en.wikipedia.org/wiki/Nucleoside>

Deoxyribonucleic Acid <http://en.wikipedia.org/wiki/DNA>